REMARKS

Following entry of the foregoing amendments, claims 14-16, 18-20, and 22-34 constitute the pending claims in the present application. Claims 1-11 and 13 are withdrawn. Claim 12 has been cancelled. Claims 32-34 are new. Support for claim 32 can be found in the specification on page 10, lines 11-12 and page 12, lines 5-6. Support for claim 33 can be found in the specification on page 10, lines 11-12 and page 12, lines 7-8 and lines 10-13. Support for claim 34 can be found in the specification on page 10, lines 12-14 and page 12, lines 6-7 and lines 9-10. Applicants assert that new claims 32-34 present no new matter.

Applicants appreciate the helpful suggestions made by the Examiner during a telephonic conference on October 5, 2004. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

1. Claim Rejections - 35 U.S.C. 112, first paragraph

Claims 14-16 and 22-29 are rejected under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants respectfully traverse this rejection to the extent it is maintained over the claims as amended.

The Office Action repeats the previous allegation that while the specification is enabled for a method of preparing a composition comprising the therapeutic agents set forth in claim 18, a complexing agent set forth in claim 20, and a polymer that is cyclodextrin, the specification does not reasonably provide enablement for a method of preparing a composition comprising the step of: combining a therapeutic agent, a polymer having host and/or guest functionality, and a complexing agent to form the composition.

In regard to Applicants' arguments in the reply to the previous Office Action, the Examiner has alleged that "the specification and the examples do not provide sufficient disclosure that would provide one of ordinary skill guidance to practice the invention, given the infinite amount of possible permutations of the claimed elements." The Office Action has

asserted that "undue experimentation is required in order to select appropriate element in order to produce a composition with *the desired effect*" (emphasis added).

Applicants respectfully disagree and bring to the Examiner's attention that the instant invention is directed toward a method of *preparing* a composition which is, at its heart, a delivery vehicle for a therapeutic agent. Applicants submit that the elements of the claimed composition are well understood and fully enabled within the current teachings of the art. One such element of the composition is a polymer having host and/or guest functionality. The Examiner has acknowledged that the instant claims are enabled for a polymer that comprises cyclodextrin. Although Applicants submit that the original claims are fully enabled for reasons already made of record, Applicants have amended the instant claims to recite a cyclodextrin-containing polymer solely to expedite prosecution. Applicants maintain that the other elements are fully enabled throughout their scope.

For example, the identity of the specific therapeutic agent used to practice the invention is *not critical*, since the nature of the therapeutic agent is relevant only to the disease or condition that is to be treated and requires *no specialized interactions* with the other components of the claimed compositions. One of skill in the art determines the identity of the therapeutic agent of the instant invention using the expansive knowledge of therapeutics for targeted diseases well known to those in the art. Determining the appropriate therapeutic for treating a particular disease is routine to one of skill in the art. Thus, the selection of components of the composition is properly left to the skilled artisan, and a corresponding rejection for lack of enablement is untenable.

Simply put, one of skill in the art would not require undue experimentation to practice the invention, since specific therapeutics and their targeted diseases were well known at the time of filing. Practicing the instant invention, preparing a composition, does not require judicious selection of the therapeutic agent with respect to the other elements of the composition; rather, *any* suitable therapeutic for a disease of interest may be selected. As a result, there would have been no undue experimentation for one of skill in the art to choose a therapeutic agent at the time the application was filed.

Similarly, another element of the composition is a complexing agent that forms an inclusion complex with the cyclodextrin-containing polymer. One of skill in the art, especially in light of the instruction provided by the instant disclosure, would also readily be able to discriminate which complexing agents can form an inclusion complex with a cyclodextrin-containing polymer. As noted above for selection of the therapeutic element of the invention, the particular identity of the cyclodextrin-containing polymer and the complexing agent are *not* not determinative of success or failure in practicing the instant invention, provided that the cyclodextrin-containing polymer and said complexing agent form an inclusion complex. Thus one of skill in the art, armed with the vast knowledge in the field on host-guest interactions (see Exhibit A from Applicants' reply of May 28, 2004 and paragraphs 105 and 106 of the specification), would readily be able to select a complexing agent that forms an inclusion complex with a cyclodextrin-containing polymer.

The basis for an enablement rejection due to "undue experimentation...in order to select appropriate element in order to produce a composition with the *desired effect*" (emphasis added) is unsound because the claimed invention does not purport to produce a composition which produces any particular "desired effect." It is the practitioner of the invention, one of ordinary skill in the art, who will determine the components of the composition, and hence a "desired effect," using knowledge readily available in the art at the time of filing. In the case of the complexing agent, the practitioner can make selections based on knowledge in the art to modify and adjust the properties of the composition. The Examiner has stated that the complexing agent "also contains a functional poup [sic] which adds a beneficial property to the composition of the invention." Again, this is a parameter that can be tailored by the practitioner for whatever purpose is intended using knowledge already readily available in the art.

For example, the complexing agent and/or functional group can be varied to optimize the bioavailability, solubility, or release rate of the composition, cyclodextrin-containing polymer, and/or therapeutic agent. Techniques and strategies for tuning these and other pharmacokinetic properties were well documented in the current literature at the time of filing. For example, in addition to the medicinal chemists' staple, *Burger's Medicinal Chemistry and Drug Discovery, 5th ed. Vols 1-6*, Burger, A.; New York: Wiley 1995, please see Exhibit A (pages 13-23 of Silverman, R. B. 1992. "The Organic Chemistry of Drug Design and Drug Action." Academic

Press, San Diego), which describes several strategies of for structural modification of compounds to optimize pharmacokinetic properties. See also Exhibit B (Harris et al. *Clin Pharmacokinet*, **2001**, *40*, 539-551), which reviews the state of the art, before the filing of the instant application, in modifying pharmacokinetic properties of therapeutic molecules through attachment of poly(ethylene glycol). These techniques, although described for modifying the properties of the drugs themselves, are the same types of modifications taught by the instant application for modifying the properties of the polymeric composition. It is Applicants who have combined these teachings in the art in preparing a composition that employs a cyclodextrin-containing polymer. The complexing agent has no specialized interactions with the other components of the composition other than to form an inclusion complex with the cyclodextrin-containing polymer. Rather, the complexing agent is selected to interact with components of the external environment, whether it is merely to modulate the solubility in a biological fluid, or to target the composition to a particular biological binding partner.

The particular complexing agent merely fine-tunes the properties of the composition and is not determinative of the success or failure of the composition in delivering the therapeutic to a patient. The Office Action provides no reasoning to the contrary, no evidence or argument that would suggest that a skilled artisan would encounter difficulty successfully preparing an operative drug-delivery composition in practicing the instant invention. Accordingly, the rejected claims are not *prima facie* non-enabled based on "undue experimentation...required in order to select appropriate element in order to produce a composition with the desired effect."

The Office Action states that "In the field of chemistry generally, there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim." Applicants assert that preparing a composition as set forth in the pending claims, e.g., combining a cyclodextrin-containing polymer having host and/or guest functionality, a therapeutic agent, and a complexing agent, does not involve chemical reactions at all, but rather involves merely mixing the three components; the specific identities of the components do not determine successful mixing. It is clear that just because there is a possibility for a large number of compositions to be derived from selection of different components, this

does not render the instant method of preparation claims *prima facie* non-enabled since the component selection process is not critical to achieving success in preparing the composition.

> Pursuant to MPEP 2164.04:
>
> [I]n order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention....As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370 (emphasis in original).

Applicants submit that the Examiner has not met the burden to provide basis for a rejection for lack of enablement. The Examiner has provided *no evidence* demonstrating that any therapeutic agent, any cyclodextrin-containing polymer, and any complexing agent cannot be used in the instant invention. Only the unsupported and conclusory assertion that "given the infinite amount of possible permutations...undue experimentation is required in order to select appropriate element in order to produce a composition with the desired effect" is presented. As noted above, this assertion is inapposite since the present invention is not limited to any particular "desired effect."

For the reasons presented above, Applicants maintain that the specification fully enables the scope of the pending claims given the highly developed state of cyclodextrin host-guest chemistry at the time of filing, the common knowledge of selecting therapeutic agents for a particular disease to be treated, and the available techniques in optimization of pharmacokinetic properties. Accordingly, one of skill in the art could readily prepare an operative composition as set forth in the pending claims without undue experimentation. Accordingly, Applicants submit that claims 14-16 and 22-29 fully comply with 35 U.S.C. 112, first paragraph. Applicants respectfully request reconsideration and withdrawal of this rejection.

In passing, Applicants note the Office Action's statement regarding a statement made by Applicants that is "on its face, contrary to generally accepted scientific principles." Applicants

are unclear as to the meaning and reference of this statement and, if this rejection is to be maintained, respectfully request that the Examiner clarify what aspect of the composition is "contrary to generally accepted scientific principles." Additionally, Applicants note that on page 4, the Office Action states that "the specification does not offer any guidance on how one of ordinary skill would go about practicing the invention for *recovery* of every claimed therapeutic agent, a polymer having host and/or guest functionality, and complexing agent." (emphasis added). Applicants are unclear as to how "recovery" is relevant to the claimed invention and respectfully request clarification if this rejection is to be maintained.

2. Claim Rejections - 35 U.S.C. 112, first paragraph

Claim 20 is rejected under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Applicants respectfully traverse this rejection to the extent it is maintained over the claims as amended.

The Office Action states that the specification "does no more than describe the desired *function* of the compound called for, that is, it does not clearly set forth the structure of the desired compounds." Moreover, the Office Action states that "the claimed 'functional group' contains almost no information by which a person of skill in the art would understand that the inventors possessed the claimed invention."

Applicants contend that the specification makes clear that the functional group serves to "add a beneficial property to the composition of the invention" (see page 39, lines 14-16), to improve or modulate the already viable composition. As is true for other elements of the instant composition, a wide variety of functional groups for modifying the properties of polymers were well known to those of skill in the art at the time of filing. The selection of a particular functional group is not dictated by the present invention. Rather, the practitioner of the invention, one of skill in the art possessing at least a Ph.D. in organic or medicinal chemistry, determines the identity of the functional group based on her intended purpose for the composition. Whereas the instant invention discloses a method of preparing the composition, the selection of the elements of the composition, such as the functional group, is premised on knowledge already in the art.

The Office Action makes reference to *Eli Lilly & Co.*, 119 F.3d 1568: "In claims involving [non-genetic] chemical materials, generic formulae usually indicate with specificity what the generic claims encompass..." However, Applicants assert that the issue in *Eli Lilly* was whether the disclosure conveys to one of ordinary skill in the art what the Applicant believed she invented, where the thing being claimed is new and not known in the art. In the instant case, the invention is a method of preparing a composition. The individual elements of the composition are not new; it is the way they are assembled and combined that is new. Applicants assert that a functional group is one element of this composition, and the functional group identity is readily determined by the practitioner of the invention from knowledge already existing in the art. Furthermore:

> *Eli Lilly* did not hold that all functional descriptions ... necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.

Moba, B.V v. Diamond Automation, Inc., 325 F.3d 1306 (Fed. Cir. 2003). Accordingly, Applicants maintain that the written description requirement is satisfied since one of skill in the art would have known many functional groups that would possess desired properties, such as hydrophobicity (hydrocarbons), hydrophilicity (polar and charged groups), ionic binding (charged groups), hydrogen bonding capability (hydroxyl, amine, amide groups, etc.), avidin affinity (biotin), increased solubility (polyethylene glycol), etc. In fact, the instant specification provides numerous examples of functional groups such as these, including hydrophobic and hydrophilic groups, hydroxyl and amine groups, ligands, nuclear localization signals, endosomal release peptides and polymers, membrane permeabilization agents, solubilizing polymers, etc. (see pages 39-41). Considering the extensive guidance available in the art at the time of filing and the supplemental teachings by the instant disclosure, one of ordinary skill in the art would have recognized that Applicants had possession of the claimed invention. Reconsideration and withdrawal of the rejection is respectfully requested.

3. Rejection based on 35 U.S.C. 103 (a) over Agrawal

Claims 14, 18-20, and 22-31 are rejected as being unpatentable over U.S. Patent 5,691,316 (Agrawal). Applicants respectfully traverse this rejection.

Pursuant to MPEP 2142:

> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicants' disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Agrawal describes "a composition comprising adamantane which is *covalently* linked to an oligonucleotide phosphorothiolate or oligonucleotide phosphodiester and non-covalently complexed with a cyclodextrin" (emphasis added). While the use of a therapeutic agent, an adamantane, and cyclodextrin are contemplated, Agrawal provides no suggestion or motivation for one of skill in the art to prepare a composition comprising a therapeutic agent that is a separate molecule from an inclusion complex comprising a complexing agent and a *polymer* having host and/or guest functionality. This deficiency in the cited reference was presented previously by Applicants, yet the Examiner still has not addressed the fact that Agrawal teaches a composition including a cyclodextrin monomer, whereas the instant claims recite a cyclodextrin-containing polymer. A cyclodextrin-containing polymer is not obvious in light of a cyclodextrin monomer, and the Office Action lacks any evidence or reasoning to the contrary. Thus, Agrawal does not teach all the elements of the instant invention. Additionally, one of skill in the art would have realized that large differences in chemical and physical properties can exist between polymers and their corresponding monomers. Since it is well established that a compound and its properties are inseparable (See *In re Papesch*, 315 F.2d 381, 137 U.S.P.Q. 43 (C.C.P.A 1963)), modification of a monomer to its corresponding polymer is unobvious because each can have different properties from the other. Given these considerations, one of skill in the art would have no motivation or expectation of success in modifying the cyclodextrin monomer of Agrawal to a cyclodextrin-containing polymer.

Furthermore, Applicants assert that Agrawal provides no suggestion or motivation for one of skill in the art to prepare a composition comprising a therapeutic agent that is a *separate molecule* from an inclusion complex comprising a complexing agent and a polymer having host and/or guest functionality. To emphasize the importance of the covalent linkage between adamantane and the therapeutic agent (oligonucleotide phosphorothiolate), Agrawal describes experiments directed to determining whether linkage of the cyclodextrin-associated oligonucleotide to adamantane had an effect on their uptake into cells (column 9, line 29). Cells were treated for varying amounts of time with fluorescently labeled oligonucleotide, fluorescently labeled cyclodextrin associated nucleotide, or fluorescently labeled covalently-linked adamantane/oligonucleotide. In the presence of cyclodextrin, the increase is much more dramatic, with the increase being the greatest with adamantane-linked oligonucleotide. Thus, covalent linkage of oligonucleotides to adamantane enhances the cellular uptake of cyclodextrin-associated oligonucleotides. Given that Agrawal teaches that superior results are a consequence of a *covalent linkage* between the adamantane and the oligonucleotide in comparison to a non-covalent association of cyclodextrin and oligonucleotide, there would have been *no motivation* or *expectation of success* in using a therapeutic agent that is a *separate molecule* from the adamantane and cyclodextrin. Furthermore, Agrawal does not even teach a composition comprising a therapeutic agent (oligonucleotide) that is a *separate molecule* from an inclusion complex (i.e., cyclodextrin and non-covalently linked adamantane); that is, the composition of oligonucleotide, cyclodextrin, and adamantane, wherein all elements are separate molecules, is never disclosed. Hence, Applicants submit that Agrawal *does not teach all of the elements* of the instant claims.

The Office Action has dismissed the above arguments in asserting that "absent objective evidence to the contrary, one of ordinary skill would expect that at least a portion of the oligonucleotide phosphorothiolate or oligonucleotide phosphodiester would be non-covalently linked, or unbound, and therefore meet applicant's claim." Applicants disagree and respectfully direct the Examiner's attention to column 12, lines 42-67 of Agrawal. The phosphodiester-linked adamantane-associated oligonucleotides are synthesized using solid phase chemistry on CPG beads. It was routine in the art to wash the solid phase products, which include the covalently bound oligonucleotide-adamantane species, after reactions to remove any unbound reagents, such as those claimed by the Examiner. Agrawal performs such a purification step (see column

12, line 32-33, reference to Damha et al.). Furthermore, the covalently linked oligonucleotide-adamantane species of Agrawal are then subjected to two deprotection steps (line 50 and 54), each of which is followed by a chromatographic purification step. Thus there are *at least three* separate purification steps subsequent to the covalent linkage of the oligonucleotide and adamantane moiety which would remove the non-covalently linked oligonucleotide and adamantane impurities mentioned by the Examiner. There is no indication in Agrawal of incompetence with regard to these purification steps. Thus, one of skill in the art would not be motivated by the teachings of Agrawal or have a reasonable expectation of success in preparing a non-covalently bound oligonucleotide-adamantane composition, in particular because Agrawal takes meticulous steps to remove impurities such as these, which further suggests their undesirability. Indeed, Agrawal et al. only achieve the purpose of their invention through the covalent linkage; unbound nucleic acid would be wasted if not deleterious.

Additionally, the Office's assumed inherency of the non-covalently bound oligonucleotide in the products synthesized by Agrawal is legally unfounded. Pursuant to MPEP § 2112, "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is *necessarily* present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.'" (emphasis added) *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d, 1949, 1950-51 (Fed. Cir. 1999). Applicants assert that this is clearly not the case, as one of ordinary skill in the art would readily accept that Agrawal is successful in eliminating the unreacted non-covalently bound oligonucleotide and adamantane species after a minimum of three routine purification steps. One of skill in the art would certainly not recognize that the "missing descriptive matter," the non-covalently linked oligonucleotide and adamantane species, would *necessarily* be present in the Agrawal products subsequent to so many purification steps.

> Additionally, pursuant to MPEP 2112:
> "In relying on the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis in original).

The Examiner has not provided any such basis and/or technical reasoning to rely on inherency in supporting the obviousness rejection. Only the baseless conclusion - apparently resting on the assumption that sloppy laboratory techniques are widespread in the art - that "absent objective evidence to the contrary, one of ordinary skill would expect that at least a portion of the oligonucleotide phosphorothiolate or oligonucleotide phosphodiester would be non-covalently linked, or unbound, and therefore meet applicant's claim" is offered. This unsupported allegation is insufficient to meet the high standard of the word "necessarily" and the rigorous requirements of an inherency argument. Thus, Applicants maintain that the Office has not demonstrated that the instant claims are *prima facie* obvious in light of Agrawal.

For the reasons set forth above, Applicants submit that *none* of the criteria for establishing a *prima facie* case of obviousness have been satisfied. Accordingly, it is believed that the pending claims satisfy the requirements of U.S.C. 103(a). Reconsideration and withdrawal of this rejection are respectfully requested.

4. Rejection based on 35 U.S.C. 103 (a) over Agrawal

Claims 15 and 16 are rejected as being unpatentable over U.S. Patent 5,691,316 (Agrawal). Applicants respectfully traverse this rejection.

Claims 15 and 16 are directed to a method of preparing a composition comprising combining a therapeutic agent, a polymer having host and/or guest functionality, and a complexing agent to form the composition, wherein said polymer and said complexing agent form an inclusion complex and said complexing agent, polymer, and therapeutic agent are combined in a particular order to form the composition.

For the reasons set forth above with respect to the independent claims from which these claims depend, Applicants submit that *none* of the criteria for establishing a *prima facie* case of obviousness have been satisfied. Accordingly, it is believed that the pending claims satisfy the requirements of U.S.C. 103(a). Reconsideration and withdrawal of these rejections are respectfully requested.

Copending Application

Applicants respectfully bring to the Examiner's attention rejections made in related co-pending US application 10/021,294.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,

Date: November 23, 2004

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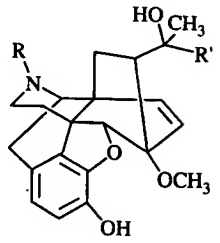
The Organic Chemistry of
Drug Design and Drug Action
Richard B. Silverman
Academic Press, San Diego: 1992

In some cases an increase in structural complexity and/or rigidity can lead to increased potency. For example, an oripavine derivative such as etorphine (**2.26**, R = CH_3, R′ = C_3H_7), which has a two-carbon bridge and a substituent not in morphine, is about 1000 times more potent than morphine[16] and, therefore, is used in veterinary medicine to immobilize large animals. The related analog, buprenorphine (**2.26**, R = CH_2—  , R′ = *tert*-Bu, double bond reduced) is 10–20 times more potent than morphine and has a very low level of dependence liability. Apparently, the additional rigidity of the oripavine derivatives increases the appropriate receptor interactions (see Chapter 3).

Once the pharmacophore is identified, manipulation of functional groups becomes consequential.

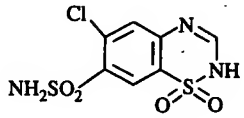


2.26

B. Functional Group Modification

The importance of functional group modification was seen in Section I,B,4; the amino group of carbutamide (**2.12**, R = NH_2) was replaced by a methyl group to give tolbutamide (**2.12**, R = CH_3), and in so doing the antibacterial activity was separated away from the antidiabetic activity. In some cases an experienced medicinal chemist knows what functional group will elicit a particular effect. Chlorothiazide (**2.27**) is an antihypertensive agent that has a strong diuretic (increased urine excretion) effect as well. It was known from sulfanilamide work that the sulfonamide side chain can give diuretic activity (see Section II,C). Consequently, diazoxide (**2.28**) was prepared as an antihypertensive drug without diuretic activity.

There, obviously, is a relationship between the molecular structure of a compound and its activity. This phenomenon was first realized over 120 years ago.



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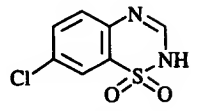


2.28

EXHIBIT A

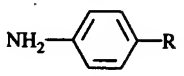
C. Structure–Activity Relationships

In 1868 Crum-Brown and Fraser,[17] suspecting that the quaternary ammonium character of curare may be responsible for its muscular paralytic properties, examined the neuromuscular blocking effects of a variety of simple quaternary ammonium salts and quaternized alkaloids in animals. From these studies they concluded that the physiological action of a molecule was a function of its chemical constitution. Shortly thereafter, Richardson[18] noted that the hypnotic activity of aliphatic alcohols was a function of their molecular weight. These observations are the basis for future structure–activity relationships (SAR).

Drugs can be classified as being structurally specific or structurally nonspecific. *Structurally specific drugs*, which most drugs are, act at specific sites, such as a receptor or an enzyme. Their activity and potency are very susceptible to small changes in chemical structure; molecules with similar biological activities tend to have common structural features. *Structurally nonspecific drugs* have no specific site of action and usually have lower potency. Similar biological activities may occur with a variety of structures. Examples of these drugs are gaseous anesthetics, sedatives and hypnotics, and many antiseptics and disinfectants.

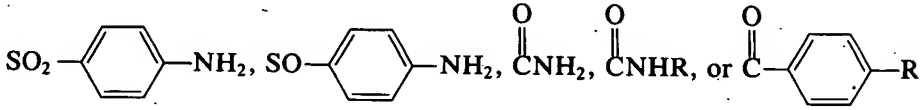
Even though only a part of the molecule may be associated with the activity, there are a multitude of molecular modifications that could be made. Early SAR studies (prior to the 1960s) simply involved the syntheses of as many analogs as possible of the lead and their testing to determine the effect of structure on activity (or potency). Once enough analogs were prepared and sufficient data accumulated, conclusions could be made regarding structure–activity relationships.

An excellent example of this approach came from the development of the sulfonamide antibacterial agents (sulfa drugs). After a number of analogs of the lead compound sulfanilamide (**2.1**, R = H) were prepared, it was found that compounds of this general structure exhibited diuretic and antidiabetic activities as well as antimicrobial activity. Compounds with each type of activity eventually were shown to possess certain structural features in common. On the basis of the biological results of greater than 10,000 compounds, several SAR generalizations have been made.[19] Antimicrobial agents have structure **2.29** (R = SO_2NHR' or SO_3H) where (1) the amino and sulfonyl groups on the benzene ring should be para; (2) the anilino amino group may be unsubstituted (as shown) or may have a substituent that is removed *in vivo*; (3) replacement of the benzene ring by other ring systems, or the introduction of



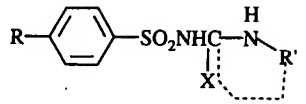
2.29

additional substituents on it, decreases the potency or abolishes the activity; (4) R may be



but the potency is reduced in most cases; (5) N′-monosubstitution (R = SO_2NHR') results in more potent compounds, and the potency increases with heteroaromatic substitution; and (6) N′-disubstitution (R = $SO_2NR'_2$), in general, leads to inactive compounds.

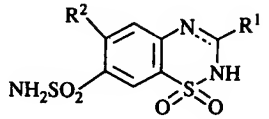
Antidiabetic agents are compounds with structure **2.30**, where X may be O, S, or N incorporated into a heteroaromatic structure such as a thiadiazole or a pyrimidine or in an acyclic structure such as a urea or thiourea. In the case of ureas, the N^2 should carry as a substituent a chain of at least two carbon atoms.[20]



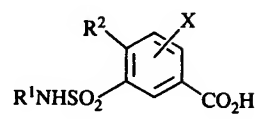
2.30

Sulfonamide diuretics are of two general structural types, hydrochlorothiazides (**2.31**) and the high ceiling type (**2.32**). The former compounds have 1,3-disulfamyl groups on the benzene ring, and R^2 is an electronegative group such as Cl, CF_3, or NHR. The high ceiling compounds contain 1-sulfamyl-3-carboxy groups. Substituent R^2 is Cl, Ph, or PhZ, where Z may be O, S, CO, or NH, and X can be at position 2 or 3 and is normally NHR, OR, or SR.[21]

The sulfonamide example is strong evidence to support the notion that a correlation does exist between structure and activity, but how do you know what molecular modifications to make in order to fine-tune the lead compound?



2.31



2.32

D. Structure Modifications to Increase Potency and Therapeutic Index

In the preceding section it was made clear that structure modifications were the keys to activity and potency manipulations. After years of structure–activity relationship studies, various standard molecular modification ap-

proaches have been developed for the systematic improvement of the *therapeutic index* (also called the *therapeutic ratio*), which is a measure of the ratio of undesirable to desirable drug effects. For *in vivo* systems the therapeutic index could be the ratio of the LD_{50} (the lethal dose for 50% of the test animals) to the ED_{50} (the effective dose that produces the maximum therapeutic effect in 50% of the test animals). The larger the therapeutic index, the greater the margin of safety of the compound. A number of these structural modification methodologies follow.

1. Homologation

A *homologous series* is a group of compounds that differ by a constant unit, generally a CH_2 group. As will become more apparent in Section II,E, biological properties of homologous compounds show regularities of increase and decrease. For many series of compounds, lengthening of a saturated carbon side chain from one (methyl) to five to nine atoms (pentyl to nonyl) produces an increase in pharmacological effects; further lengthening results in a sudden decrease in potency (Fig. 2.1). In Section II,E,2,b it will be shown that this phenomenon corresponds to increased lipophilicity of the molecule, which permits penetration into cell membranes until its lowered water solubility becomes problematic in its transport through aqueous media. In the case of aliphatic amines another problem is micelle formation, which begins at about C_{12}. This effectively removes the compound from potential interaction with the appropriate receptors. One of, if not the, earliest example of this potency versus chain length phenomenon was reported by Richardson,[18] who was

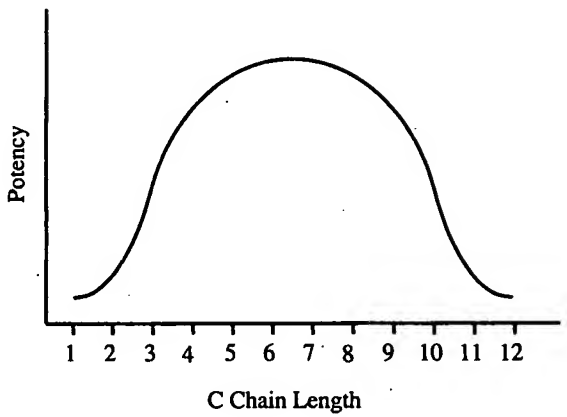
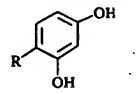
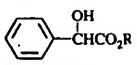


Figure 2.1. General effect of carbon chain length on drug potency.

Table 2.1 Effect of Chain Length on Potency: Antibacterial Activity of 4-*n*-Alkylresorcinols[22a] and Spasmolytic Activity of Mandelate Esters[22b]





R	Phenol coefficient	% Spasmolytic activity[a]
methyl	—	0.3
ethyl	—	0.7
n-propyl	5	2.4
n-butyl	22	9.8
n-pentyl	33	28
n-hexyl	51	35
n-heptyl	30	51
n-octyl	0	130
n-nonyl	0	190
n-decyl	0	37
n-undecyl	0	22
i-propyl	—	0.9
i-butyl	15.2	8.3
i-amyl	23.8	28
i-hexyl	27	—

[a] Relative to 3,3,5-trimethylcyclohexanol, set at 100%.

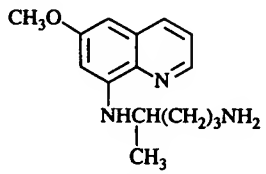
investigating the hypnotic activity of alcohols. The maximum effect occurred for 1-hexanol to 1-octanol; then the potency declined upon chain lengthening until no activity was observed for hexadecanol.

A study by Dohme *et al.*[22a] on 4-alkyl-substituted resorcinol derivatives showed that the peak antibacterial activity occurred with 4-*n*-hexylresorcinol (see Table 2.1), a compound now used as a topical anesthetic in a variety of throat lozenges. Funcke *et al.*[22b] found that the peak spasmolytic activity of a series of mandelate esters occurred with the *n*-nonyl ester (see Table 2.1).

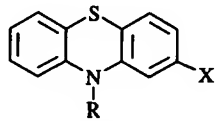
2. Chain Branching

When a simple lipophilic relationship is important as described above, then *chain branching* lowers the potency of a compound. This phenomenon is exemplified by the lower potency of the compounds having isoalkyl chains in Table 2.1. Chain branching also can interfere with receptor binding. For example, phenethylamine ($PhCH_2CH_2NH_2$) is an excellent substrate for monoamine oxidase [amine oxidase (flavin-containing)], but α-methylphenethylamine (amphetamine) is a poor substrate. Primary amines often are more potent than secondary amines which are more potent than tertiary amines. For example, the antimalarial drug primaquine (**2.33**) is much more potent than its secondary or tertiary amine homologs.

Major pharmacological changes can occur with chain branching and homologation. Consider the 10-aminoalkylphenothiazines (**2.34**, X = H). When R is $CH_2CH(CH_3)N(CH_3)_2$ (promethazine) or $CH_2CH_2N(CH_3)_2$ (diethazine), antispasmodic and antihistaminic activities predominate. However, the homolog **2.34** with R being $CH_2CH_2CH_2N(CH_3)_2$ (promazine) has greatly reduced antispasmodic and antihistaminic activities, but sedative and tranquilizing activities are greatly enhanced. In the case of the branched chain analog **2.34** with R equal to $CH_2CH(CH_3)CH_2N(CH_3)_2$ (trimeprazine), the tranquilizing activity is reduced and antipruritic (anti-itch) activity increases.



2.33



2.34

3. Ring–Chain Transformations

Another modification that can be made is the transformation of alkyl substituents into cyclic analogs. Consider the promazines again (**2.34**). Chlorpromazine [**2.34**, X = Cl, R = $CH_2CH_2CH_2N(CH_3)_2$] and **2.34** (X = Cl, R = $CH_2CH_2CH_2N$⬠) are equivalent as tranquilizers in animal tests. Trimeprazine [**2.34**, X = H, R = $CH_2CH(CH_3)CH_2N(CH_3)_2$] and methdilazine [**2.34**, X = H, R = CH_2—CH(—CH_2—CH_2)—N—CH_3] have similar antipruritic activity in man.

Different activities can result from a ring–chain transformation as well. For example, if the dimethylamino group of chlorpromazine is substituted by a methylpiperazine ring (**2.34**, X = Cl, R = $CH_2CH_2CH_2N$⬡NCH_3; prochlorperazine), the antiemetic (prevents nausea and vomiting) activity is greatly enhanced. In this case, however, an additional amino group is added.

4. Bioisosterism

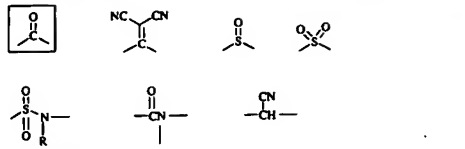
Bioisosteres are substituents or groups that have chemical or physical similarities, and which produce broadly similar biological properties.[23] Bioisosterism is a lead modification approach that has been shown to be useful to attenuate toxicity or to modify the activity of a lead, and it may have a significant role in the alteration of metabolism of a lead. There are classical isosteres[24,25] and nonclassical isosteres.[23,26] In 1925 Grimm[27] formulated the *hydride displacement law* to describe similarities between groups that have the same number of valence electrons but may have a different number of atoms. Erlenmeyer[28] later redefined isosteres as atoms, ions, or molecules in which the peripheral layers of electrons can be considered to be identical. These two definitions describe *classical isosteres*; examples are shown in Table 2.2. *Nonclassical*

Table 2.2 Classical Isosteres[24,25]

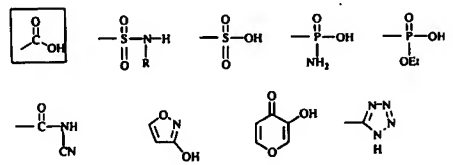
1. Univalent atoms and groups					
a. CH_3	NH_2	OH	F	Cl	
b. Cl	PH_2	SH			
c. Br	*i*-Pr				
d. I	*t*-Bu				
2. Bivalent atoms and groups					
a. $—CH_2—$	—NH—	—O—	—S—	—Se—	
b. $—COCH_2R$	—CONHR	$—CO_2R$	—COSR		
3. Trivalent atoms and groups					
a. —CH=	—N=				
b. —P=	—As=				
4. Tetravalent atoms					
a. $—\overset{|}{\underset{|}{C}}—$	$—\overset{|}{\underset{|}{Si}}—$				
b. =C=	$=\overset{+}{N}=$	$=\overset{+}{P}=$			
5. Ring equivalents					
a. —CH=CH—	—S—	(e.g., benzene, thiophene)			
b. —CH=	—N=	(e.g., benzene, pyridine)			
c. —O—	—S—	$—CH_2—$	—NH—	(e.g., tetrahydrofuran, tetrahydrothiophene, cyclopentane, pyrrolidine)	

Table 2.3 Nonclassical Bioisosteres[23]

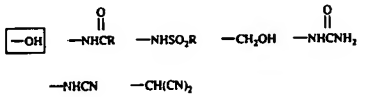
1. **Carbonyl group**



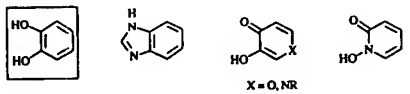
2. **Carboxylic acid group**



3. **Hydroxy group**

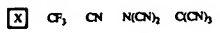


4. **Catechol**

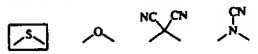


X = O, NR

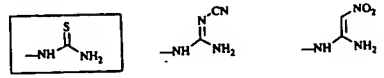
5. **Halogen**



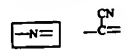
6. **Thioether**



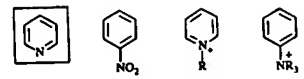
7. **Thiourea**



8. **Azomethine**



9. **Pyridine**

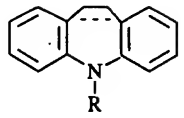


10. **Spacer group**

11. **Hydrogen**

bioisosteres do not have the same number of atoms and do not fit the steric and electronic rules of the classical isosteres, but they do produce a similarity in biological activity. Examples of these are shown in Table 2.3.

Ring–chain transformations also can be considered to be isosteric interchanges. There are hundreds of examples of compounds that differ by a bioisosteric interchange[23,26]; some examples are shown in Table 2.4. Bioisosterism also can lead to changes in activity. If the sulfur atom of the phenothiazine neuroleptic drugs (**2.34**) is replaced by the —CH=CH— or —CH_2CH_2— bioisosteres, then dibenzazepine antidepressant drugs (**2.35**) result.



2.35

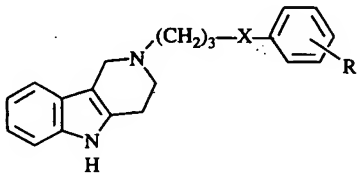
It is, actually, quite surprising that bioisosterism should be such a successful approach to lead modification. Perusal of Table 2.2, and especially of Table 2.3, makes it clear that in making a bioisosteric replacement, one or more of the following parameters will change: size, shape, electronic distribution, lipid solubility, water solubility, pK_a, chemical reactivity, and hydrogen bonding. Because a drug must get to the site of action, then interact with it (see Chapter 3), modifications made to a molecule may have one or more of the following effects:

1. Structural. If the moiety that is replaced by a bioisostere has a structural role in holding other functionalities in a particular geometry, then size, shape, and hydrogen bonding will be important.
2. Receptor interactions. If the moiety replaced is involved in a specific interaction with a receptor or enzyme, then all of the parameters except lipid and water solubility will be important.
3. Pharmacokinetics. If the moiety replaced is necessary for absorption, transport, and excretion (collectively, with metabolism, termed *pharmacokinetics*) of the compound, then lipophilicity, hydrophilicity, pK_a, and hydrogen bonding will be important.
4. Metabolism. If the moiety replaced is involved in blocking or aiding metabolism, then the chemical reactivity will be important.

It is because of these subtle changes that bioisosterism is effective. This approach allows the medicinal chemist to tinker with only some of the parameters in order to augment the potency, selectivity, and duration of action and to reduce toxicity. Multiple alterations may be necessary to counterbalance effects. For example, if modification of a functionality involved in binding also decreases the lipophilicity of the molecule, thereby reducing its ability to

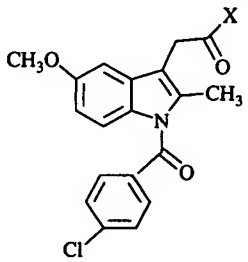
Table 2.4 Examples of Bioisosteric Analogs[23,26]

1. **Neuroleptics (antipsychotics)**



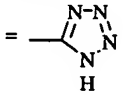
X=  or CHCN

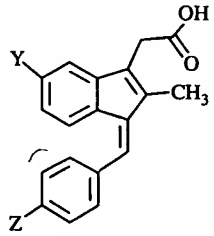
2. **Anti-inflammatory agents**



X = OH (indomethacin)

= NHOH

= 



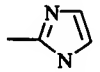
Y = CH_3O Z = Cl

Y = F Z = SCH_3 (sulindac)

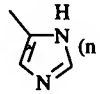
3. **Antihistamines**

R — X — $(CH_2)_n$ — Y X = NH, O, CH_2

Y = $N(CH_3)_2$ (n = 2)



(n = 1)



(n = 1, 2)

penetrate cell walls and cross other membranes, the molecule can be substituted with additional lipophilic groups at sites distant from that involved with binding. Modifications of this sort may change the overall molecular shape and result in another activity.

Up to this point we have been discussing more or less random molecular modifications to make qualitative differences in a lead. In 1868 Crum-Brown and Fraser[17] predicted that some day a mathematical relationship between structure and activity would be expressed. It was not until almost 100 years later that this prediction began to be realized and a new era in drug design was born. In 1962 Corwin Hansch attempted to quantify the effects of particular substituent modifications, and from this quantitative structure–activity relationship (QSAR) studies developed.[29]

E. Quantitative Structure–Activity Relationships

1. Historical

The concept of quantitative drug design is based on the fact that the biological properties of a compound are a function of its *physicochemical parameters*, that is, physical properties, such as solubility, lipophilicity, electronic effects, ionization, and stereochemistry, that have a profound influence on the chemistry of the compounds. The first attempt to relate a physicochemical parameter to a pharmacological effect was reported in 1893 by Richet.[30] He observed that the narcotic action of a group of organic compounds was inversely related to their water solubility (Richet's rule). Overton[31] and Meyer[32] related tadpole narcosis induced by a series of nonionized compounds added to the water in which the tadpoles were swimming to the ability of the compounds to partition between oil and water. These early observations regarding the depressant action of structurally nonspecific drugs were rationalized by Ferguson.[33] He reasoned that, for a state of equilibrium, simple thermodynamic principles could be applied to drug activities, and that the important parameter for correlation of narcotic activities was the relative saturation (termed *thermodynamic activity* by Ferguson) of the drug in the external phase or extracellular fluids. This is known as *Ferguson's principle*, which is useful for the classification of the general mode of action of a drug and for predicting the degree of its biological effect. The numerical range of the thermodynamic activity for structurally nonspecific drugs is 0.01 to 1.0, indicating that they are active only at relatively high concentrations. Structurally specific drugs have thermodynamic activities considerably less than 0.01 and normally below 0.001.

In 1951 Hansch *et al.*[34] noted a correlation between the plant growth activity of phenoxyacetic acid derivatives and the electron density at the ortho position (lower electron density gave increased activity). They made an at-

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0312-5963/01/0007-0539/$22.00/0

Pegylation

A Novel Process for Modifying Pharmacokinetics

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Contents

Abstract

The use of liposomal carriers and the modification of therapeutic molecules through the attachment of poly(ethylene glycol) [PEG] moieties ('pegylation') are the most common approaches for enhancing the delivery of parenteral agents. Although 'classical' liposomes (i.e. phospholipid bilayer vehicles) have been effective in decreasing the clearance of encapsulated agents and in passively targeting specific tissues, they are associated with considerable limitations.

Pegylation may be an effective method of delivering therapeutic proteins and modifying their pharmacokinetic properties, in turn modifying pharmacodynamics, via a mechanism dependent on altered binding properties of the native protein. Pegylation reduces renal clearance and, for some products, results in a more sustained absorption after subcutaneous administration as well as restricted distribution. These pharmacokinetic changes may result in more constant and sustained plasma concentrations, which can lead to increases in clinical effectiveness when the desired effects are concentration-dependent.

Maintaining drug concentrations at or near a target concentration for an extended period of time is often clinically advantageous, and is particularly useful in antiviral therapy, since constant antiviral pressure should prevent replication and may thereby suppress the emergence of resistant variants. Additionally, PEG modification may decrease adverse effects caused by the large variations in peak-to-trough plasma drug concentrations associated with frequent administration and by the immunogenicity of unmodified proteins. Pegylated proteins may have

reduced immunogenicity because PEG-induced steric hindrance can prevent immune recognition.

Two PEG-modified proteins are currently approved by the US Food and Drug Administration; several others, including cytokines such as interferon-α (IFNα), growth factors and free radical scavengers, are under development. Careful assessment of various pegylated IFNα products suggests that pegylated molecules can be differentiated on the basis of their pharmacokinetic properties and related changes in pharmacodynamics. Because the size, geometry and attachment site of the PEG moiety play a crucial role in determining these properties, therapeutically optimised agents must be designed on a protein-by-protein basis.

Numerous strategies have been evaluated in an attempt to improve the delivery of pharmaceutical agents. Drug delivery can be modified either through a change in formulation or by a change in molecular structure. Novel drug formulation modifications, such as colloidal systems (e.g. liposomes, microspheres) and continuous release mechanisms (e.g. osmotic pumps) can provide benefits over standard formulations, particularly when long-lasting drug concentrations are achieved.[1] In contrast to modifying the formulation of a drug, chemical attachment of poly(ethylene glycol) [PEG] moieties to therapeutic compounds (a process known as 'pegylation') represents a new approach that may enhance important drug properties.

Although innovative formulations of oral medications are numerous, providing extended release delivery of intravenous drugs, particularly proteins, has been more difficult. Proteins and peptides are used for a number of diagnostic, monitoring and treatment applications. Parenteral administration of these agents does not, however, guarantee that adequate drug concentrations will be achieved at the site of action.[2] The optimal use of these agents is also limited by poor shelf stability, short half-lives and a potential for immunogenicity. In many instances, proteins must be administered frequently to be effective, which may increase cost, inconvenience and the risk of adverse reactions.[3]

Therapeutic proteins are rapidly cleared from the blood by the liver, kidneys and other organs via a number of mechanisms, including through the reticuloendothelial system, through specific cell-protein interactions, through renal filtration, or by proteolytic enzymes. Clearance depends on the ionic charge, molecular weight and the presence of cellular receptors.[4] Formulation changes (e.g. liposomes, microspheres, hydrogels and monoclonal antibodies) have been investigated to modify the molecular and biochemical characteristics of proteins.[3,5,6]

'Classical' liposomes (phospholipid bilayer vehicles) have been shown to alter biodistribution by reducing drug clearance, decreasing the volume of distribution (Vd) and shifting the distribution in favour of diseased tissues that have increased capillary permeability.[5] Still, liposomal delivery has a number of limitations. Liposomal particles are rapidly sequestered into the liver, spleen, kidneys and reticuloendothelial system and have a tendency to 'leak' drug while in circulation.[7] In addition, liposomes can induce complement activation, resulting in enhanced clearance as well as a risk of cardiovascular and haematological adverse events.[8] Many of these deficiencies of classical liposomes can be improved by pegylation.[5,7]

Pegylation was first developed by Davis, Abuchowski and colleagues[9] in the 1970s. Their goal was to enhance the delivery of therapeutic molecules; perhaps more importantly, pegylation has also been shown to change the pharmacokinetics and, thus, the pharmacodynamics of the therapeutic molecule without the limitations of classical liposomes.[3,10] The pharmacokinetic modifications produced in pegylated proteins, as compared with their unmodified counterparts, have prompted the investigation of this technology for a number of therapeutic applications. Although protein-PEG

conjugates have generated the most interest and are the primary focus of this review, a variety of molecules can be conjugated to PEG (table I). The advantage of using PEG conjugation for nonprotein molecules is primarily related to increased water solubility, reduced renal clearance and decreased toxicity.[10]

1. Principles of Pegylation

PEG moieties are inert, long-chain amphiphilic molecules produced by linking repeating units of ethylene oxide. A large number of potential PEG molecules are available, and they can be produced in different configurations, including linear or branched structures, and in different molecular weights (fig. 1). Using pegylation to increase the size and molecular weight of a therapeutic protein alters the immunological, pharmacokinetic and pharmacodynamic properties of the protein in ways that can extend its potential uses.[11,12]

Goals for chemically coupling polymers to peptide and protein drugs include decreased clearance, retention of biological activity, obtaining a stable

Table I. Potential types of poly(ethylene glycol) conjugates[10]

Conjugate type	Properties and applications
Small molecule drugs	Improved solubility, controlled permeability through biological barriers, longevity in bloodstream, controlled release
Affinity ligands and cofactors	Used in aqueous 2-phase partitioning systems for purification and analysis of biological macromolecules and cells. Enzymatic reactors
Peptides	Improved solubility, conformational analysis, biologically active conjugates
Proteins	Resistance to proteolysis, reduced immunogenicity and antigenicity, longevity in bloodstream, tolerance induction. Uses: therapeutics, organic soluble reagents, bioreactors
Saccharides	New biomaterials, drug carriers
Oligonucleotides	Improved solubility, resistance to nucleases, cell membrane permeability
Lipids	Used for preparation of PEG-grafted liposomes
Liposomes and particulates	Longevity in bloodstream, RES-evasion
Biomaterials	Reduced thrombogenicity, reduced protein and cell adherence

PEG = poly(ethylene glycol); **RES** = reticuloendothelial system.

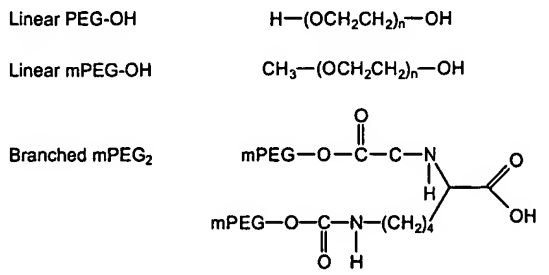

Fig. 1. Structural formulae of poly(ethylene glycol) [PEG] molecules.[11] **mPEG** = monomethoxypoly(ethylene glycol).

linkage, and enhanced water solubility without significantly altering bioavailability (for example by subcutaneous injection). These changes can produce a number of clinical advantages, such as sustained plasma concentrations, decreased adverse effects, improved patient convenience and enhanced quality of life. Sustained plasma concentrations may contribute to increased effectiveness when the desired effects are concentration- and time-dependent.

2. Process of Pegylation

Characteristics of both the PEG moiety and the native protein affect the manufacturing approach to developing a pegylated protein with pharmacokinetic and pharmacodynamic properties superior to the unmodified protein.

The average size of the attached PEG moiety, as well as the total number of available attachment sites on the protein, contribute to the size and the net total molecular weight of the conjugated protein. Large proteins generally have more attachment sites and, therefore, are commonly multipegylated. Attachment at multiple sites, however, increases the likelihood of steric interference at the active site of the native protein, resulting in a possible inhibition or reduction of activity. The attachment of branched PEG moieties can increase the size of the moiety (and net total molecular weight of the conjugated protein) without a resultant increase in the number of attachment sites. In addition, branched chain PEG conjugates have been shown to have increased pH and thermal stability

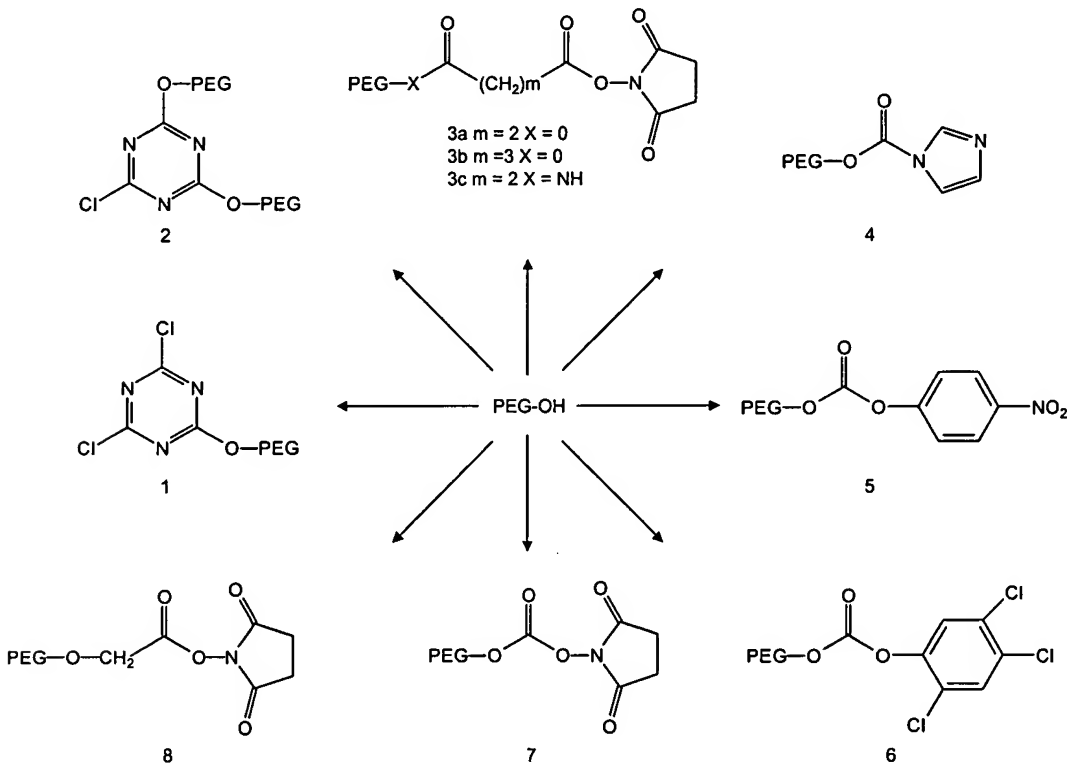

Fig. 2. Method for the activation of poly(ethylene glycol) [PEG] molecules.[15] (1) Trichloro-s-triazine (cyanuric chloride) method; (2) a variation of the cyanuric chloride method; (3a) PEG-succinimidyl succinate method; (3b) substitution of the succinate residue by glutarate; (3c) substitution of the aliphatic ester in 3a by an amide bond; (4) imidazolyl formate method; (5) and (6) are variations using phenylcarbonates of PEG; (7) succinimidyl carbonates of PEG; (8) succinimidyl active esters of PEG. **mPEG** = monomethoxypoly(ethylene glycol).

and increased resistance to proteolytic digestion compared with linear PEG conjugates.[13] Small proteins generally have fewer attachment sites and can be effectively pegylated with a single large (possibly branch-chained) PEG moiety.

A stable linkage between the PEG moiety and the drug is important to ensure that PEG-induced pharmacological changes are maintained. Protein pegylation is generally achieved by formation of linkages between an amino group on the protein and an active carbonate, active ester, aldehyde or tresylate derivative of PEG.[14] PEG-bearing chemically reactive groups are synthesised through chemical modification of the terminal hydroxyl groups of the native PEG moiety.[10] The PEG moiety is activated through this substitution of the hydroxyl group by an electrophilic functional group (fig. 2).[15] The PEG molecule can be made monofunctional by use of methoxy-PEG, as this PEG form has a single hydroxyl group for activation and because the methoxy group is inert to standard chemical processes.[16]

The reactive functional group of activated PEG can then be attached to a specific site (e.g. amine, sulphydryl group or other nucleophile) on the therapeutic molecule. In the majority of cases, covalent attachment of PEG derivatives utilises amino groups of lysines and the N-terminus of polypeptide molecules as the site of modification.[15] PEG derivatives suitable for amine modification include *N*-hydroxysuccinimidyl-activated esters (producing an amide linkage), PEG-epoxide (amine linkage),

PEG-carbonyl imidazole (urethane linkage), PEG-tresylate (amine linkage) and PEG-aldehyde (amine linkage). Thiol groups such as protein cysteine groups can be modified by use of PEG-maleimide and vinyl sulfone, among others.[10,11,17,18]

Varying pegylation chemistries or reaction conditions can result in differences in the functional properties of therapeutic proteins. For example, PEG conjugation of granulocyte colony-stimulating factor through alkylation with PEG-aldehyde produced increased stability compared with conjugation through acylation with an active ester.[19] In part, this was the result of selectivity by aldehyde for reaction with the N-terminus.

For poorly reactive reactants, increasing the pH, temperature, reagent-protein molar ratio and reaction time may be required to obtain the desired degree of PEG substitution (e.g. mono-, di-, tri-conjugates).[11,17]

In a typical protein pegylation via reaction with lysine and N-terminal amines, PEG attaches to one or more of several potential sites on the protein, each attachment location defining a different isotype. The distribution of PEG isotypes has interesting implications in the drug development process. The product must be defined by the distribution specifications because the activity of the product is a function of the defined mixture. Consistency of the distribution of PEG isotypes must be demonstrated across the entire drug development programme, including process changes and scale-up. Each development programme must establish specifications for the distribution of these isotypes based on the frequency of the isotypes noted in material used for pivotal clinical trials. As long as specifications are established and consistency can be demonstrated, characterisation of the activity of the individual PEG isotypes is not relevant and is typically not required by regulatory agencies.

3. Effect of Pegylation on Pharmacokinetic and Pharmacodynamic Properties

Pegylation increases the size and molecular weight of a molecule. It also produces alterations in the physicochemical properties of the parent molecule. These include changes in conformation, steric hindrance, changes in electrostatic binding properties, hydrophobicity, local lysine basicity and pI (the pH at which a protein's charge is neutral).[11] These physical and chemical changes reduce systemic clearance by a number of mechanisms, including decreases in renal clearance, proteolysis and opsonisation (macrophage uptake),[20] and can influence the binding affinity of the therapeutic protein to cellular receptors, resulting in changes in the bioactivity of the agent.[17] In addition, pegylation may increase the absorption half-life of subcutaneously administered agents, and is sometimes associated with a decreased Vd.[21]

A PEG mass of approximately 40 to 50kD is required to retard the glomerular filtration of small molecules.[11] Smaller molecules are freely filtered at various rates.[12] This threshold size can be achieved either by attaching a large PEG moiety at a single site or attaching several small PEG moieties at more than one site. It is also noteworthy that the PEG molecule is heavily hydrated and in rapid kinetic motion, so the 'effective' molecular weight of PEG is greater than its apparent molecular weight.[10]

Pegylation may decrease cellular protein clearance by reducing elimination through the reticuloendothelial system or by specific cell-protein interactions.[20] In addition, pegylation forms a protective 'shell' around the protein. This shell and its associated waters of hydration shield the protein from immunogenic recognition and increase resistance to degradation by proteolytic enzymes, such as trypsin, chymotrypsin and *Streptomyces griseus* protease.[3,12] For example, pegylation reduced the degradation of asparaginase by trypsin: after a 50-minute incubation period, there was 5, 25 and 98% residual activity of native asparaginase, PEG-asparaginase and branched-PEG-asparaginase, respectively (fig. 3).[13]

The decreased clearance effected by pegylation, alone or in combination with targeted drug delivery systems, can be used to alter distribution or even to increase the delivery of therapeutic mole-

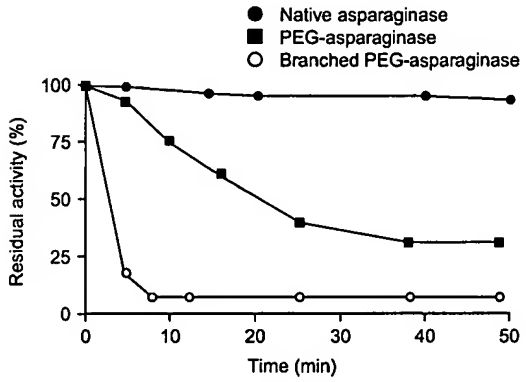

Fig. 3. Time course of hydrolysis of native asparaginase, PEG-asparaginase and branched PEG-asparaginase by trypsin as assessed by enzyme activity.[13] PEG = poly(ethylene glycol).

cules to specific sites. For example, the delivery of brain-derived neurotrophic factor (BDNF) across the blood-brain barrier was enhanced through the combined use of pegylation and a tethered monoclonal antibody (OX26 monoclonal antibody to the transferrin receptor, which undergoes receptor-mediated transcytosis through the blood-brain barrier).[22] Pegylation of BDNF minimised the rapid clearance of the peptide and allowed for enhanced drug delivery through the blood-brain barrier. Animal studies have shown that varying the size of the PEG moiety can alter the distribution of pegylated interferons in tissue; indeed, these studies indicated that increasing PEG molecular weight decreased renal clearance while increasing hepatic clearance.[23]

A number of strategies can be used to optimise the pharmacological characteristics of pegylated proteins. Excessive pegylation may decrease the activity of a protein.[24] In general, a single PEG moiety is more likely to conserve biological activity, especially when the activity depends on interaction with another macromolecule, although the activity of certain enzymes often survives multiple pegylations.[25] Some researchers have reported an inverse relationship between PEG mass and *in vitro* activity but a direct relationship between PEG mass and *in vivo* activity (fig. 4). The activity of pegylated compounds in *in vitro* assays is decreased because pegylated molecules typically demonstrate decreased receptor binding as a result of steric interference.

Increasing the molecular weight of the PEG moiety, however, also decreases the clearance of the therapeutic molecule, thereby producing sustained exposure to the agent. This prolonged presence of the active agent may negate or overcome the decrease in receptor binding. Thus, when the biological activity of a pegylated biopharmaceutical agent is measured *in vivo*, one frequently observes a direct relationship between the mass of the PEG conjugate and its biological activity, with increased mass associated with increased activity. A threshold to this increase can also be observed. Increasing the mass of the PEG conjugate beyond this threshold value negatively alters its molecular characteristics.[17]

Table II summarises the effect of pegylation on the pharmacokinetic and pharmacodynamic properties of several therapeutic proteins. Pegylation of interleukin-6 (IL-6) produced a 100-fold increase in half-life, resulting in a 500-fold increase in thrombopoietic potency, and a decrease in immune

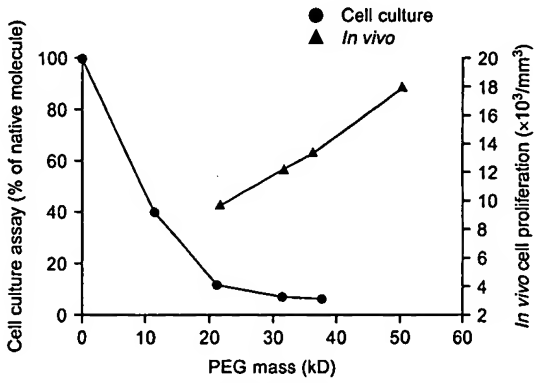

Fig. 4. *In vitro* and *in vivo* bioactivity of a cytokine (RO-cytok-1) as a function of PEG mass (kD). *In vitro* assay: M-NFS-60 cell proliferation assay measures the incorporation of [³H]thymidine into cellular DNA during the final 6 hours of cell culture. *In vivo* assay: female C57BL/6J murine model used for subcutaneous injection of drug. Venous blood withdrawn on the fifth day after injection. Differential leukocyte counts were determined from blood smears (Wright stain) [reproduced from Bailon and Berthold[11] and Fung et al.,[17] with permission]. PEG = poly(ethylene glycol).

response as evidenced by reduced plasma IgG1 and adverse effects compared with the native compound in mice.[31] A marked decrease in uptake of IL-6 by the reticuloendothelial system was also demonstrated.[31] The same investigators also showed that pegylation of tumour necrosis factor increased antitumour potency and decreased toxic effects in the murine fibrosarcoma model.[32] In other animal models, pegylated analogues of growth hormone-releasing factor produced an increase in the area

Table II. Influence of pegylation on pharmacokinetics and/or pharmacodynamics of therapeutic proteins

Pharmacokinetic effect[a]	Pharmacodynamic effect[a]	References
Interferon-α-2a		
Sustained absorption (subcutaneous first order absorption half-life ↑ from 2.3 to 50h[b]	*In vitro* antiviral activity increased 12- to 135-fold	26-30
Terminal $t_{1/2\beta}$ ↑ (from 3-8h to 65h)[b]	Antitumour activity increased 18-fold	
Vd ↓ (from 31-73L to 8-12L)[b]	Improved sustained responses in chronic hepatitis C	
CL ↓ 100-fold (from 6.6-29.2 to 0.06-0.10 L/h)[b]		
Interleukin-6		
$t_{1/2\beta}$ ↑ 100-fold (from 2.1 to 206 min)	Thrombopoietic potency increased 500-fold	31
	Decreased IgG1 production	
Tumour necrosis factor		
$t_{1/2\beta}$ ↑ 14- to 43-fold (from 3 to 45-136 min)	Antitumour potency increased 4- to 100-fold	32
	Decreased adverse events	
Growth hormone-releasing factor		
	Potency increased 12- to 55-fold, enhanced duration of action	33
Megakaryocyte growth and development factor		
$t_{1/2\beta}$ ↑ 10-fold	*In vitro* activity increased 20-fold	34
Brain-derived neurotrophic factor		
$t_{1/2\beta}$ ↑ 5-fold (from 10 to 50 min)		22
CL ↓ 2.6-fold (from 5.5 to 1.9 ml/min/kg)		
Vd ↑ 1.7-fold (from 80 to 135 ml/kg)		
Asparaginase		
$t_{1/2\beta}$ ↑ 18-fold (from 20 to 357h)		35
Vd unchanged		
AUC ↑ 26-fold (from 0.4 to 10.2 IU/ml/day)		
CL ↓ 17-fold (from 2196 to 128 ml/m^2/day)		
Superoxide dismutase		
$t_{1/2\beta}$ ↑ >150-fold (from 3.5 to 540-990 min)		36
Lactoferrin		
$t_{1/2\beta}$ ↑ 5- to 20-fold (from 3 to 15-60 min)		36
Streptokinase		
$t_{1/2\beta}$ ↑ 1.7- to 5-fold (from 4 to 7-20 min)	Decreased antigenicity	37
Interleukin-2		
$t_{1/2\beta}$ ↑ up to 6-fold (from 44 to 57-256 min)		38
CL ↓ up to 10-fold (from 1.15 to 0.11 to 0.97 ml/min)		
Vd unchanged		

a Compared with native protein.

b Unmodified interferon compared with pegylated interferon [branched PEG(40kD)-IFNα-2a; higher end of the range reported for different pegylated interferons].

AUC = area under the plasma concentration-time curve; **CL** = systemic clearance; $t_{1/2\beta}$ = terminal elimination half-life; **Vd** = volume of distribution.

under the growth hormone concentration-time curve and a prolonged duration of action compared with the native compound, presumably because of a decrease in clearance; these changes represent the potential for a decrease in dosage of up to 20-fold (in mice) to 50-fold (in pigs).[33]

4. Effect of Pegylation on Immunogenicity and Adverse Effects

One of the major drawbacks of using proteins of nonhuman origin for therapeutic purposes is the risk of immunogenic and antigenic responses. These responses can neutralise biological activity, increase elimination of the protein and result in hypersensitivity reactions.[12] The development of immunogenicity occurs more frequently when the protein is administered subcutaneously. Pegylation of proteins appears to lower the immunogenic response by steric masking of potential antigenic sites, thus preventing immune recognition of the therapeutic protein as foreign (see reviews by Nucci et al.[3] and Delgado et al.[12]).

The benign nature of PEG molecules has been validated through their long history of use. Studies involving the administration of PEG (molecular weight >400) to a wide variety of species have demonstrated no significant toxicity.[39] PEG is approved by the US Food and Drug Administration (FDA) for use as a vehicle or base in a variety of pharmaceutical preparations, including injectable, topical, rectal, and nasal products, as well as in foods and cosmetics.[3,10]

Pegylation has the potential to decrease adverse effects of the therapeutic molecules they are attached to and, thus, to increase patient compliance and improve quality of life. For example, in a murine model, pegylation of IL-6 not only resulted in an augmentation of thrombopoietic activity, but also reduced IL-6–induced plasmacytosis, piloerection and hypoalbuminaemia.[31] The decrease in adverse effects may reflect a decrease in the fluctuations in plasma concentrations caused by frequent, high doses of unmodified protein, or a reduction in the immunogenic response as a result of fewer administrations of the pegylated protein and steric masking of antigenic sites.

One recent study has suggested that PEG-linked proteins have the potential to induce renal tubular vacuolation in an animal model.[40] Rats treated with PEG-linked tumour necrosis factor binding factor developed vacuolation of the renal cortical tubular epithelium. It is likely that this effect is unique to this native protein and dependent on the molecular weight of the attached PEG moiety. The change was associated with extremely high doses and not accompanied by alterations of clinical pathology or functional markers. The effect was also inversely proportional to the molecular weight of the PEG: the lowest molecular weight PEG conjugate was associated with the most severe vacuolar changes, and animals treated with higher molecular weight compounds had minimal or no lesions.[40]

5. Pegylated Liposomes

Liposomes can be modified with PEG to prolong their blood circulation time. Pegylated liposomes are characterised by an increased half-life, decreased plasma clearance and a restricted Vd compared with classical liposomal preparations. The incorporation of PEG into the lipid bilayer attracts a hydrated shell around the liposome and protects the bilayer from plasma proteins and lipoproteins, producing an 8-fold increase in plasma half-life of the liposome compared with an unmodified liposome.[41] Pegylated liposomes are also less extensively taken up by cells of the reticuloendothelial system and are less likely to leak drug while in circulation.[7]

Doxorubicin encapsulation in PEG-coated liposomes alters the pharmacokinetic characteristics and, potentially, the safety and tolerability profile of the drug.[7] The goal of this modification is to decrease toxicities such as nausea/vomiting, alopecia and cardiotoxicity compared with standard doxorubicin preparations. Such modifications also have the potential to increase tumour response due to enhanced drug accumulation in tumour cells.[7,42-46] Active targeting of tumours through the use of pegylated antitumour antibodies is also under investigation.[45]

6. Therapeutic Applications of Pegylated Drugs

Two pegylated molecules, PEG-adenosine deaminase (pegademase) and PEG-asparaginase (pegaspargase) [both products of Enzon Corp., Piscataway, NJ, USA], have been approved for clinical use in the US. In addition, several other PEG conjugates are currently under clinical development.

6.1 Pegademase

Severe combined immunodeficiency disease (SCID) is associated with an inherited deficiency of adenosine deaminase (ADA). A pegylated version of the bovine form of this enzyme, pegademase, was approved in 1990 as replacement therapy for patients who are ADA-deficient with SCID.[46] Pegademase is produced by coupling ADA to multiple strands of monomethoxypoly(ethylene glycol) 5000. Coupling with PEG increases the circulating half-life of the enzyme from a few minutes to approximately 24 hours.[9,47] Following a single intramuscular injection of pegademase, peak plasma concentrations are achieved within 2 to 3 days.[48] Pegylation of ADA also inhibits uptake of the enzyme by cells.

Maintenance of high plasma ADA concentrations reverses the principal intracellular abnormalities caused by deoxyadenosine when nucleosides equilibrate rapidly across cell membranes: nucleoside pool expansion and inactivation of *S*-adenosylhomocysteine hydrolase (SAHase).[49] Monitoring of plasma ADA activity, the levels of nucleosides and SAHase in red blood cells, and immune function enabled the establishment of optimal dose and administration frequency for PEG-ADA, and is used in individual patients to adjust dosage during therapy.[49] ADA deficiency is a rare syndrome; as of 1997, 63 patients had been treated with PEG-ADA.[48]

Before the availability of pegademase, partial exchange transfusions with red blood cells that contain ADA were used to treat ADA-deficient SCID. However, ADA activity remains elevated for only 2 to 4 weeks after transfusion. In addition, transfusions carry a risk of iron overload and transfusion-related viral infections.[4,50] Pegademase has approximately 1800-fold more ADA activity per millilitre than red blood cells; therefore, the drug produces higher concentrations of ADA activity than partial exchange transfusion.[49]

Although the development of IgG antibody to bovine ADA-specific epitopes is detectable in over half of SCID patients treated with pegademase, antibody titre does not correlate with plasma ADA concentrations.[48] Only 2 anti-ADA-positive patients have shown a transiently enhanced clearance of pegademase; however, ADA activity was restored by a short course of corticosteroids in 1 patient and by dose modifications in the other.[48] In addition, the drug has been well-tolerated, with no allergic or serious adverse effects noted after several years of use.[48,49]

6.2 Pegaspargase

Asparaginase has been a main component of treatment regimens for patients with acute leukaemias.[35] One of the major limitations of asparaginase is its tendency to induce hypersensitivity reactions and the development of neutralising antibodies that shorten the half-life, making it difficult to maintain effective plasma concentrations.[51]

Pegaspargase, formed by pegylation of the ε-amino groups on the lysine residues of asparaginase, is available for use in the US for the treatment of patients with acute lymphocytic leukaemia, acute lymphoblastic leukaemia and chronic myelogenous leukaemia. The primary advantages of pegaspargase over the unmodified compound are that it decreases the tendency to induce an immune response, allowing the majority of patients with hypersensitivity to the native enzyme to tolerate pegaspargase without further clinical hypersensitivity, and extends the half-life from the 20 hours seen with the native compound to 357 hours with the PEG-modified compound.[35] Several studies have demonstrated the efficacy of pegaspargase in the treatment of acute leukaemias, and have established current dosage recommendations.[35]

7. Pegylated Products in Development

A number of agents, primarily therapeutic proteins and enzymes, are candidates for pegylation and are under development. These include growth factors (e.g. growth hormone-releasing factor, granulocyte colony-stimulating factor), free radical scavengers (e.g. superoxide dismutase, catalase), blood derivatives (e.g. haemoglobin, albumin), antineoplastic agents (e.g. uricase, interferons, anthracyclines), cardiovascular agents (e.g. streptokinase, catalase, tissue plasminogen activator), antigens (e.g. honeybee venom, ragweed pollen) and antihepatitis C agents [e.g. interferon-α (IFNα)].[4] Because several PEG-IFNα congeners are currently under investigation, these provide an ideal case for studying the differential effect of PEG moiety size and shape on the pharmacology and clinical efficacy of a therapeutic protein. Thus, PEG-IFNα is discussed in more detail in section 8.

8. Pegylated Interferon-α

The delivery of IFNα represents a significant challenge and has potential implications in multiple therapeutic indications, including patients with hepatitis B and C infections, malignant melanoma, renal cell carcinoma, chronic myelogenous leukaemia, non-Hodgkin's lymphoma and myeloma. Following subcutaneous administration, IFNα is rapidly absorbed, with peak serum concentrations observed in 7 to 12 hours, followed by a rapid decline.[26,27,30,52] The terminal elimination half-life ($t_{½β}$) of IFNα ranges from only 3 to 8 hours, with serum concentrations decreasing to below the limit of detection within 24 hours of administration.[26,27,30] Thus, when IFNα is administered in the standard 3-times weekly regimen, serum concentrations are undetectable for much of the administration interval.

PEGs of varying lengths and shapes have been attached to IFNα-2a and IFNα-2b to optimise the protein conjugate and change the pharmacokinetics. Table III provides a conceptual overview of the differences in the pharmacokinetic properties of standard IFNα and 3 different pegylated products in clinical development. Published data are used whenever possible to illustrate how the size and branching of the PEG moiety alters the absorption, distribution and clearance of the IFNα molecule. The pharmacokinetic parameters are presented as ranges if these are reported from multiple studies.

Although the various pegylated IFNα products extend $t_{½β}$ to a similar degree, other pharmacokinetic parameters are affected differently. Only the 40kD branched pegylated IFNα-2a [PEG(40kD)-IFNα-2a] is associated with a decreased Vd and a much more sustained absorption half-life ($t_{½abs}$). The Vd of PEG(40kD)-IFNα-2a is approximately 10L (the approximate volume of the plasma and extracellu-

Table III. Pegylation alters absorption, distribution and clearance of interferon-α in a size-dependent manner

Pharmacokinetic parameter[a]	Unmodified IFNα[26,27]	Linear PEG(5kD)-IFNα-2a[21][b]	Linear PEG(12kD)-IFNα-2b[53][c]	Branched PEG(40kD)-IFNα-2a[28-30][d]
Vd (L) after an IV dose	31-73	Similar to IFNα	Similar to IFNα	8-12
CL (L/h) after an IV dose	6.6-29.2	2.5-5.0	0.725[e]	0.06-0.10
$t_{½abs}$ (h) after a SC dose	2.3	Similar to IFNα	4.6	50[f]
$t_{½β}$ (h) after an IV dose	3-8	54[e]	54[e]	65[e]
t_{max} (h) after a SC dose	7.3-12	20[e]	20[e]	80[e]

a Determined after IV administration, or values reported in the literature after SC administration were corrected for absolute bioavailability.

b Administered as single 45, 135 and 270μg SC doses.

c Administered as multiple 0.035 to 2 μg/kg SC doses once weekly for 24 weeks.

d Administered as single 45 to 270μg SC doses.

e Data not reported as a range because of the limited information available.

f Assuming first-order absorption.

CL = systemic clearance; **IV** = intravenous; **SC** = subcutaneous; **IFNα** = interferon-α; **PEG-IFN** = pegylated interferon-α; $t_{½abs}$ = absorption half-life; $t_{½β}$ = elimination half-life; t_{max} = time to maximum drug concentration; **Vd** = volume of distribution.

lar water), which is 4-fold lower than that of standard IFNα.[30]

Following subcutaneous administration, PEG-(40kD)-IFNα-2a plasma concentrations were measurable within 3 to 6 hours and reached a maximum at about 80 hours.[30] This longer time to maximum plasma concentration (t_{max}) could be related to either the sustained rate of absorption or the reduced clearance of PEG(40kD)-IFNα-2a compared with standard IFNα. The 40kD product is associated with a greater reduction in renal clearance and with more constant and sustained maximal serum concentrations than either the 5kD linear PEG-IFNα-2a [PEG(5kD)-IFNα-2a] or the 12kD linear PEG-IFNα-2b [PEG(12kD)-IFNα-2b].[21,28,29,53] There was no significant change in subcutaneous bioavailability of PEG(40kD)-IFNα-2a as compared with unmodified IFNα.[21,28-30]

The sustained absorption and reduced clearance of subcutaneously administered PEG(40kD)-IFNα-2a allows for a more convenient dosage schedule (once weekly versus 3-times weekly for standard IFNα-2a) and is probably responsible for the higher rates of sustained virological response seen in patients treated with this pegylated IFNα for chronic hepatitis C virus infection. Monotherapy with standard IFNα is associated with a sustained biological response in only 15 to 25% of patients,[54,55] and PEG(5kD)-IFNα-2a was no more effective than standard IFNα.[56] In contrast, results from randomised clinical trials indicate that PEG(40kD)-IFNα-2a produces much higher end-of-treatment and sustained virological responses than standard IFNα in patients with chronic hepatitis C virus infection, with or without cirrhosis.[57-59] For example, the sustained virological response rate in patients without cirrhosis was twice as great in patients treated with PEG(40kD)-IFNα-2a as in those treated with standard IFNα (39% *vs* 19%).[58] Thus, PEG(40kD)-IFNα-2a may represent an advance in the treatment of chronic hepatitis C virus infection.

Initial multiple dose pharmacokinetic studies for PEG(40kD)-IFNα-2a showed that the changes in sustained absorption, reduced clearance and restricted distribution of PEG(40kD)-IFNα-2a result in predictable and sustained (near-constant) concentrations with peak : trough ratios of 1.3 to 2.0.[60] Using a 1-compartment open model with first order absorption and elimination, the single dose pharmacokinetic data listed in table III, and the recommended dose and administration interval for each agent, the predicted peak : trough ratios were 1.5 for PEG(40kD)-IFNα-2a, 6 for PEG-(12kD)-IFNα-2b, 20 to 40 for PEG(5kD)-IFNα-2a, and more than 40 for standard IFNα.

Large fluctuations in serum IFNα concentrations (i.e. high peak : trough ratios) may explain why titres of hepatitis C virus rebound quickly between doses of standard IFNα.[29,61] Constant antiviral pressure should prevent viral rebound and continued viral replication, and may decrease the potential for emergence of resistant quasispecies.

9. Conclusions

Pegylation now represents a promising sustained-action delivery method for injectable medications. Pegylation of therapeutic proteins and peptides can reduce immunogenicity and modify the pharmacokinetics and, hence, change the pharmacodynamics of these agents while maintaining subcutaneous bioavailability. Thus, pegylation may overcome many of the pharmacological limitations of therapeutic proteins. The potential advantages of pegylation include increased circulating exposure to the therapeutic protein, decreased acute adverse effects, more convenient dosage regimens, and increased health-related quality of life.

Because all proteins are different, each PEG moiety must be specifically optimised to the targeted therapeutic molecule. The length and shape of each PEG moiety are crucial in determining the effect on pharmacokinetic and pharmacodynamic properties. Pegylated agents in development must also meet standards of homogeneity and pyrogenicity, and activation and coupling techniques must be reproducible. Despite these challenges, techniques for pegylating molecules have progressed enormously in recent years.

The coupling of PEG to therapeutic proteins has revealed a wide range of possible pharmaceutical

applications, and a number of new pegylated products, such as pegylated IFNα, have shown promise for improving the pharmacodynamics, pharmacokinetics and clinical effects of therapeutic proteins. It is important, however, to note the differences between pegylated forms of the same therapeutic agent. The case of pegylated IFNs for use in the treatment of patients with chronic hepatitis C virus infection clearly demonstrates the potential for dissimilar characteristics among PEG conjugates. A careful assessment of both pharmacological and clinical properties must be considered when determining the optimal pegylated form of the therapeutic molecule for clinical utility.

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